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NOR Activity In *Chironomus Riparius* Mg. (DIPTERA, CHIRONOMIDAE) Is Affected By Chronic Exposure To Trace-Metal Pollution

Paraskeva Michailova[*], Julia Ilkova[*], Piero Cervella[§], Alfredo Santovito[§], Gabriella Sella[§]

[*] Institute of Biodiversity and Ecosystem Research - Bulgarian Academy of Sciences - 1, Tsar Osvoboditel Blvd - 1000 Sofia, Bulgaria

e-mail: michailova@zoology.bas.bg

[§] Department of Life Science and System Biology University of Turin - via Accademia Albertina 13 - 10123 Torino, Italy

Abstract

We compared the activity of the Nucleolar Organizer Region (NOR) of fourth-instar larvae of Chironomus riparius from a heavy-metal-polluted station with that of larvae from a control by means of FISH with a 28S-rDNA probe. This probe should hybridize with the 28S-rDNA genes and their transcripts, also pointing out the transcriptional activity. In addition, at the cytogenetic level we expected to find that changes of gene expression would appear as variation of nucleolar size, as indicated by FISH signals. The mean frequency of the active NORs (0.834 ± 0.10) in the larvae from the heavy-metal-polluted station was significantly lower than that in the larvae of the control (0.976 ± 0.055). These results confirm the hypothesis that trace metals reduce the level of transcription activity of the rDNA of the NORs.

Key words: Chironomidae, trace metal pollution, rDNA, Nucleolar Organizer

Introduction.

The salivary-gland polytene chromosomes of the larvae of chironomids are very suitable structures for analyzing patterns of gene expression, as the most active genetic sites can be visualized as puffs. Chromosome G of Chironomus riparius Mg. has very well visible sites of transcription corresponding to the Nucleolar Organizer Region (NOR) and the Balbiani rings (BRs). NOR can be defined as a puff in which transcription of rDNA leads to synthesis of rRNA and assembly of ribosomes. rDNA consists of hundreds of repeated genes, but only part of them are actively transcribed [1,2]. Kiknadze [3] showed that three bands of chromosome G (2b8, 2b9, 2b10) form the core of the NOR. During ontogenesis these bands undergo considerable structural changes. The NOR is actively transcribed during the 6–7 phase of the 4th larval stage [4]. At cytogenetic level, this activity is shown by the spreading of chromatin fibres in the whole NOR and by the decondensed state of the three above-quoted bands. The NOR is a very sensitive structure which undergoes functional changes after trace-metal exposure [5–7]. Planellò et al. [7] showed a retraction of rDNA fibres of NOR under 10 mM Cd acute treatment in a laboratory experiment. Therefore, the hypothesis has been advanced that NORs can be considered as very sensitive biomarkers for trace-metal pollution [6, 7]. In larvae from a heavy-metal-polluted station of Chaya River (near Asenovgrad, Bulgaria), the frequency of NORs with reduced activity significantly increased compared to that of NORs of the control larvae (G test, G = 77.4, d.f. = 1, $P < 0.001$) [8].

Monitoring of NOR activity can play a key role in environmental monitoring and subsequent development of risk-assessment strategies. Therefore, we analyzed the effects of chronic heavy metal pollution on rDNA transcription in polytene chromosomes G of Chironomus riparius larvae. To this aim, the frequency of the active NORs of Chaya River

larvae was compared with that of larvae from a control. We performed Fluorescent in situ Hybridization (FISH) by using a 28S-rDNA probe. We expected that this probe would hybridize with the 28S-rDNA genes and indicate the transcription activity.

Material and methods.

Sixty-four larvae of C. riparius (at the 6–7 phase of the 4th larval stage) were used. Thirty-seven C. riparius larvae were collected from the polluted Chaya River in October 2010. Chemical analysis showed that sediments of this river contained Cu, Pb and Cd ions [8, 9] in concentrations 12–40 times higher than those reported in reference data [10]. As a control we used twenty-seven C. riparius larvae of a laboratory culture. Control larvae were reared under standard conditions (temperature 18–20 ◦C, photoperiod 16 h light and 8 h dark, constant aeration), fed twice a week and bred in pure, mash filtre paper.

Cytogenetic method. The conventional acetic-orcein method for preparing salivary gland polytene-chromosome preparations was applied for identifying C. riparius larvae collected from the field. The species-specific cytogenetic markers in the polytene chromosomes were identified [11, 12]. Standard chromosome map of the chromosome G of C.riparius done by Kiknadze [3]was used for mapping the 28S-rDNA FISH signals.

Fluorescent in situ hybridization. A probe for 28S-rDNA of C. riparius was used. This probe is a 500 bp amplicon obtained using the following primers: Cr28S-F 5′-TGAGCACACTGGATACGACCCGAA and Cr28SS-R 5′-TGTCCACCGTCCTGCTGTCTTCA. The amplicon product was obtained by amplifying approximately 20 ng genomic DNA in a final volume of 30μL, containing 1x Subtherm Taq-DNA polymerase buffer (Fisher), 1.5 mM $MgCl_2$, 0.2 μM each primer, 0.2 mM dNTPs and 1u of Subtherm Taq-DNA polymerase (Fisher) under the following conditions: 3′ of initial denaturation at 95 ◦C; 30 cycles at 94◦C for 30”, 58◦C for 30”, 72◦C for 1′, 72◦C for 5′. An aliquot was reamplified and labelled under the same conditions as above, substituting 0.05 mM TTP with 0.05 mM Digoxigenin-11-dUTP. FISH signals were detected using the anti-digoxigenin antibody (Roche) according to the protocols of Schmidt [13] and Hankeln et al. [14].

Statistical methods. The numbers of sampled larvae and examined cells are shown on Table 1. Frequencies of active NORs in the cells were not normally distributed. Therefore, we compared frequencies of the active NORs between exposed and control larvae by means of the non-parametric Wilcoxon–Mann–Whitney test [15]. The analysis was performed using the SYSTAT software, version 10.0.

Results and discussion.

All the C. riparius larvae we analyzed had a chromosome set 2n = 8 and belonged to the thummi (cytocomplex) [16], with chromosome arm combinations AB CD EF G, species-specific cytogenetic marker and chromosome G carrying three Balbiani rings (BRa, BRb, BRc) and a Nucleolar Organizer (Fig. 1a). We observed variation of the NOR pattern both in cells of the larvae from Chaya River and of the control (Table 1). Changes of NOR patterns varied from a uniform decondensed structure (Fig. 1b), to a dense fibril structure (Fig. 1c) and to a granular structure with a restricted inner dense region (Fig. 1d). The first two patterns correspond to normal and slightly reduced transcriptional activity respectively, while the latter

suggests absence of any transcription. The chromosomal position of the inner dense region corresponds to the chromosomal sections where rDNA genes are located, according to Kiknadze [3].

Table 1

Number of sampled larvae, examined cells and frequencies of active and inactive NORs in larvae of *Chironomus riparius* from Chaya River station and the control

Locality	No of larvae	No of cells	Active NORs	Inactive NORs
Chaya River	37	337	280*	57
Control	27	396	375*	21

*$P = 0.015$ (Wilcoxon–Mann–Whitney test)

The mean frequency (mean ± SD) of cells with active NORs in the larvae from Chaya River station (0.834 ± 0.10) was significantly lower (P = 0.015) than that observed in the control larvae (0.976 ± 0.055). We assumed that both the larvae from Chaya River and those from the control had the same number of 28S ribosomal genes. Nevertheless, we observed a significant lower frequency of FISH spots in larvae from the polluted Chaya River compared to the control larvae. Therefore, we inferred that this effect could be ascribed to a reduced transcriptional activity in larvae chronically exposed to heavy metal pollution.
Planellò et al. [7] by means of FISH with their 28S-rDNA probe pointed out a retraction of rDNA towards the chromosomal axes. Cadmium acute exposure caused a significant decrease in rRNA among treated larvae compared to control larvae. Therefore, these authors concluded that ribosomal genes and NORs are the direct target for acute cadmium toxic- ity. Similarly, at cytogenetic and morphological levels, in NORs of many cells of larvae from Chaya River station we observed the retraction of nucleolar fibres and the condensed state of bands, supporting the hypothesis of transcriptional inhibition. However, the mechanism involved in these events is unclear. Further studies for understanding the effect of these modifications can shed light into the mechanisms controlling changes of transcription of rDNA under the action of stress agents.

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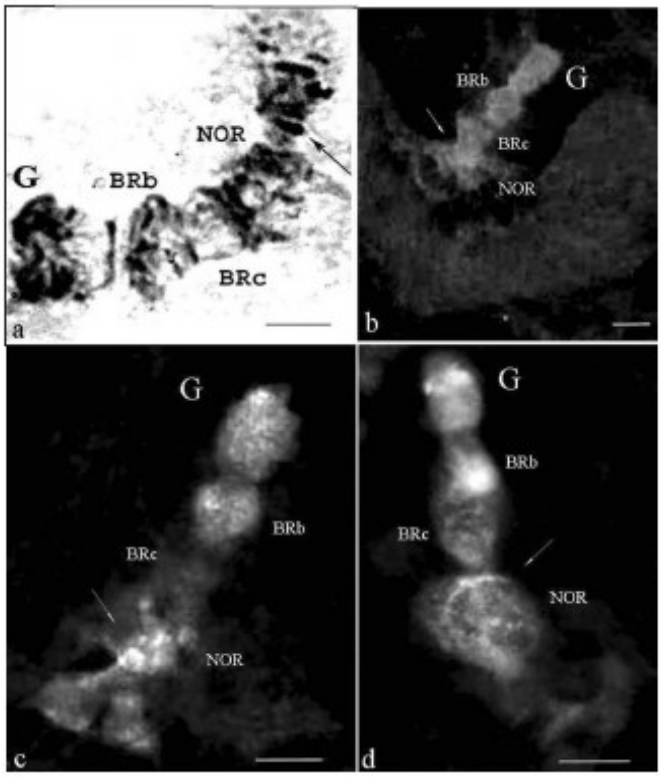

Fig 1. (a) Chromosome G of Chironomus riparius after acetic-orcein staining – standard. NOR – Nucleolar Organizer; BR – Balbiani ring; Arrow indicates the centromere localization. Chromosome G after FISH with 28S-rDNA probe
(b) nucleolar fibres are spreading all over the Nucleolar Organizer;
(c) fibril granular structure;
(d) a clearly visible condensed band.
Bar – 100µm